JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2002, p. 210–215 Vol. 40, No. 1
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.1.210–215.2002

Comparison of Six PCR Methods Using Peripheral Blood for Detection of Canine Visceral Leishmaniasis

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Received 4 April 2001/Returned for modification 8 August 2001/Accepted 14 October 2001

The objectives of this study were to compare the sensitivities and reliabilities of different PCR methods for the diagnosis and epidemiological study of canine visceral leishmaniasis (CVL) using dog blood. We chose to work with peripheral blood, as this type of sampling is noninvasive, straightforward, and easy to repeat. Six PCR methods were compared: three primer pairs target genomic DNA, and the other three target kinetoplast (mitochondrial) DNA. Sensitivity, specificity, reproducibility, and ease of interpretation without hybridization were evaluated for each method. The assessment was first performed using artificial samples. All methods could detect less than one parasite per reaction tube. However, the sensitivities varied among the different methods by a factor of 500 on purified cultivated parasites and by a factor of 10,000 on seeded dog blood samples (i.e., from 10 to 10^{-3} parasite per ml of blood for the latter). Only four methods were found sufficiently reliable for the diagnosis of CVL. They were tested on 37 dogs living in an area of endemicity and grouped according to clinical status and specific serology. Only the two methods targeting kinetoplast DNA (K13A-K13B and RV1-RV2) could detect the parasite in 100% of symptomatic infected dogs. Similarly, all seropositive dogs were found PCR positive by these methods versus 62% by the genomic-DNA-based methods. Finally, these kinetoplast-based methods proved clearly superior to the others in the detection of *Leishmania* in asymptomatic dogs. Our data allow the discussion of the advantages and drawbacks of highly sensitive versus moderately sensitive PCR methods in diagnosis and prevalence studies of CVL.

The leishmaniases are parasitic diseases widespread in the Old and New World with great epidemiological diversity. They are caused by about 20 species of *Leishmania*, protozoa transmitted by the bite of female sand flies. Visceral leishmaniasis (VL) caused by *Leishmania infantum* (syn. *Leishmania chagasi*) is a severe, often fatal, disease common in the Mediterranean region and in Latin America. Dogs are the main reservoir (22), and the disease is now recognized as a serious animal health problem (17, 36). The seroprevalence of canine leishmaniasis in areas of endemicity in the Mediterranean basin ranges between 10 and 30% (12, 15, 40, 42). However, most infected dogs do not present any clinical signs (5, 13, 15, 37), and there is evidence that the infection prevalence rates are higher than those given by serological studies (see below). Reliable diagnostic tests are therefore required for the detection of canine VL (CVL) in both clinically ill and asymptomatic dogs. The most widely used detection method is the search for specific anti-parasite antibodies in serum, which requires noninvasive sampling and yields reported sensitivities of >70% in clinically ill dogs (3, 8, 12, 29, 32). However, it is difficult to ascertain what proportion of infected dogs develop a detectable specific immune response, and studies in countries where the disease is endemic have actually shown that infected animals, particularly the asymptomatic ones, often remain seronegative or borderline positive (2, 20, 26, 38). On the whole, it appears that serology may not be a good indicator of infection (2, 6). The confirmation of the diagnosis of CVL comes from the direct observation of *Leishmania* parasites in smears and in vitro cultures of lymph node or bone marrow aspirates. This method, however, is invasive and generally unsuccessful in detecting the parasite in asymptomatic dogs (28). Several authors have shown that the use of PCR can improve the detection of CVL (2, 4, 16, 18, 31, 33, 35, 40, 43). In particular, several studies demonstrated that the proportion of asymptomatic dogs infected by the parasite was higher than previously thought. Unfortunately, like most in-house PCR assays, the PCR applied to the detection of *Leishmania* is far from standardized: most research groups have used different PCR methods based on different primer pairs with different kinds of samples. Many DNA targets have been described for "*Leishmania* PCR," but to our knowledge, very few comparative studies have been performed (19, 33), of which only one was for *L. infantum*. The selection of adequate primers is well known to be critical for both PCR sensitivity and specificity. Moreover, whereas the PCR diagnosis of VL in humans can be considered extremely efficient (7, 11, 18, 21), the PCR diagnosis of CVL continues to pose serious problems, due, for example, to our ignorance of the most informative tissue to be sampled or else to the difficulties encountered in DNA preparation or the high frequency of PCR inhibitions in the presence of dog blood.

The objectives of the present study were to compare the sensitivities of different PCR methods for the diagnosis of CVL and to analyze their respective advantages and drawbacks for routine diagnosis or epidemiological use. Six different primer pairs, all targeting repetitive DNA and previously tested in

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clinical diagnosis and field studies, were compared: three of them target nuclear genomic DNA, while the other three target the highly reiterated minicircles of kinetoplast (mitochondrial) DNA. We used peripheral blood as the source of parasites, both in the form of seeded samples and in infected dogs, for two reasons: (i) peripheral blood has been shown to be almost always PCR positive in human VL (1, 11, 27), and circulating parasites are known to occur in dogs (18, 31, 33); (ii) this type of sampling is noninvasive and straightforward compared with lymph node or bone marrow aspiration. After thorough testing using artificial samples, five and four of the six methods were tested on two groups of dogs living in an area where CVL is endemic.

MATERIALS AND METHODS

Animals. Thirty-seven dogs of different breeds and ages living in outdoor conditions in the endemic focus of the Cévennes (southern France) were recruited for the study. The CVL diagnosis was classically established from clinical signs, specific serology, and/or direct examination of lymph node or bone marrow aspirates. For serology, two techniques were used: immunofluorescence (cutoff value, 1/40) and counterimmunoelectrophoresis (cutoff value, one line). Peripheral blood for PCR was collected by jugular venipuncture in an EDTA-coated tube. A control group was made up of 10 dogs living in an area where the disease is not endemic in the north of Corrèze (France); the dogs were born there and had never left the area.

Leishmania serial dilution assay. Promastigotes from a 4-day-old culture of a reference strain of *L. infantum* (MHOM/FR/78/LEM75) were washed twice in 1× phosphate-buffered saline and precisely counted on a Thoma hemacytometer (mean of 10 counts). The pellet was lysed with proteinase K at 320 μg/ml in 2 volumes of TNNT buffer (0.5% Tween 20, 0.5% Nonidet P-40, 10 mM NaOH, 10 mM Tris, pH 7.2). The DNA was extracted by a simplified phenol-chloroform method and ethanol precipitated. A series of dilutions was performed, yielding DNA solutions corresponding to decreasing concentrations from 20 to 0.00001 parasite/μl.

Preparation of seeded blood samples. All samples consisted of 4.5 ml of peripheral blood collected in EDTA-coated tubes. The blood was collected from five healthy dogs living in an area where the disease is not endemic and was then pooled before being aliquoted again. Seeded samples were made by adding live *L. infantum* promastigotes to the buffy coat (BC) collected as described below. The concentrations of parasites tested were 1,000, 100, 10, 5, 2, and 1 per ml of whole blood, corresponding to 10, 1, 0.1, 0.05, 0.02, and 0.01 parasite per PCR. Concentrations of <1 parasite/ml of blood (i.e., <0.01 parasite/PCR) were obtained by serial dilutions of the lowest concentration given above in a solution of negative control dog blood DNA.

Sample and DNA preparation. For both seeded samples and dog samples, the BC was isolated from 4.5 ml of peripheral blood after a simple centrifugation step for 10 min at 1,600 × *g*. The BC (300 μl) was lysed in 2 volumes of TNNT buffer and 960 μl of proteinase K/ml, and the tube was incubated for between 2 and 24 h at 56°C, boiled for 10 min, and then stored at 4°C (10). Extraction lysates were processed as follows: 500 μl of lysate was subjected to phenol-chloroform extraction (i.e., phenol and phenol-chloroform followed by chloroform), the DNA was precipitated with ethanol and resuspended in 150 μl of sterile distilled water. The DNA extraction was controlled by measurement of the optical density at 260 nm in a GeneQuantII spectrophotometer (Pharmacia).

PCR amplification. Six primer pairs were compared. Their characteristics are presented in Table 1. A thorough optimization of the PCR conditions was carried out as described previously (11) for each of the PCR methods, using seeded dog blood samples instead of purified cultivated promastigotes. The optimized conditions for each method are summarized in Table 1. For all reactions, 5 μl of 10× buffer, 0.6 mg of bovine serum albumin/ml, 200 μM (each) deoxynucleoside triphosphate, and 50 pmol of primers were used in a total reaction volume of 50 μl, including 10 μl of sample DNA. The variable factors included the $MgCl_2$ concentration, the amount of *Taq* DNA polymerase (Eurogentec), and the primer annealing temperature. The "hot-start" technique (Dynawax; Eurogentec) was used routinely in all experiments to increase the technical specificity. The reactions were cycled in a Biometra thermal cycler for K13A-K13B and RV1-RV2 and in an MJResearch thermal cycler for the remaining primer pairs. The following conditions were used: initial annealing at 94°C for 4 min, 40 cycles of 94°C for 30 s, variable annealing temperature according to the primer set (Table 1) for 30 s, and 72°C for a variable duration according to the primer set, followed by a final elongation at 72°C for 10 min. Each concentration for the sensitivity assays (both with purified culture parasites and seeded blood samples) was tested at least twice in quadruplicate. The dog samples were tested twice in duplicate. Additionally, in each test, one internal positive control tube for the detection of PCR inhibition was included for each sample (11). In cases of reaction inhibition, a second DNA extraction was performed, and both DNA preparations were tested as is and diluted to 1/5 or 1/10. Finally, three to five negative control tubes that each received 10 μl of H_2O instead of DNA were included in each test to detect any carryover contamination. Uracil DNA *N*-glycosylase (UNG) was used for primers K13A-K13B and RV1-RV2: 1 U of the enzyme was added to the PCR mixture, and dTTP was replaced by dUTP (400 μM); the tube was then incubated for 10 min at 50°C prior to the first denaturation.

PCR product analysis and DNA hybridization. The reaction products were visualized under UV light after electrophoresis of 20 μl of the reaction solution in a 2 or 3% agarose gel. All gels were then Southern blotted and hybridized with the corresponding ^{32}P-labeled PCR product. The filters were washed twice at high stringency (0.1× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7], 1% sodium dodecyl sulfate) before the autoradiogram was made. Hybridization was systematically carried out for all methods. It was found necessary for methods Deb8-Ajs3 and Pia1-Pia2.

Decontamination procedures. Complete physical separation (of rooms, materials, and personnel) as well as decontamination (e.g., UV exposure of rooms, consumables, and materials and bleaching of all materials and surfaces) procedures were used routinely to avoid carryover contamination from previously amplified DNA (9, 11). Additional separation measures were taken for two primer pairs, K13A-K13B and RV1-RV2, for which the PCR and analysis of PCR products were performed in distinct hospital buildings. Enzymatic decontamination using uracil DNA *N*-glycosylase was also used with both of these primer pairs.

RESULTS

Comparison of six PCR methods with artificial *Leishmania* samples. The comparisons were based on examination of the specificity, sensitivity, ease of interpretation of results, and reproducibility of each method. The specificities of the six methods against other microorganisms had previously been tested by different authors and were not tested again here. The technical specificity was assessed from the presence of spurious amplification products revealed by artifactual bands on the electrophoresis gels. One of our positive criteria was to avoid specific DNA hybridization, which is cumbersome in routine practice. Using purified cultivated parasites, no artifactual bands were observed, except for some with Deb8-Ajs3. By contrast, the presence of dog blood DNA tended to yield spurious amplification products that hampered the interpretation of the electrophoresis gels and often made a hybridization step necessary. Great differences in this respect were observed among the different methods tested (Table 2).

Sensitivity was assessed on one hand by a *Leishmania* serial dilution assay (SDA) using cultivated parasites and on the other hand with seeded dog blood samples containing known parasite concentrations (see Materials and Methods). Using purified cultivated parasites, the sensitivities varied from 0.05 to 10^{-4} parasite per reaction tube between the least (R221-R332) and the most (K13A-K13B and RV1-RV2) sensitive methods (Table 2). The presence of blood led to a decrease in sensitivity of 10- to 100-fold with the least sensitive methods, which then detected 0.2 to 1 parasite per reaction tube. By contrast, it did not affect the sensitivities of the most sensitive methods, which detected 10^{-4} parasite per reaction tube. More pragmatically, the sensitivity thresholds varied from 10 to 10^{-3} parasite per ml of blood according to the PCR method used (Table 2). In our hands, whichever method was used, the

TABLE 1. Main characteristics of the six PCR methods used in the study

Characteristic	PCR method						
	R221-R332	Pia1-Pia2	Pia3-Pia4	Deb8-AJS3	K13A-K13B	RV1-RV2
PCR DNA target	Ribosomal DNA (20- to 40-fold repeat)	Repetitive sequence of *L. infantum* genome	Repetitive sequence of *L. infantum* genome	kDNA[b] minicircle (10,000 copies)	kDNA minicircle (10,000 copies)	kDNA minicircle (10,000 copies)
Product size (bp)	603	100	260	809	120	145
Reference(s)	41	24, 25	23	39	34	14, 30
Specificity	*Leishmania* sp.	*L. donovani* sensu lato[a]	*L. donovani* sensu lato	*Leishmania* sp.	*Leishmania* sp.	*L. donovani* sensu lato
Primer sequence	5′-GGTTCCTTTCCTGATTT ACG-3′	5′-ACGAGGTCAGCTCCAC TCC-3′	5′-CGGCTTCGCACCATGC GGTG-3′	5′-CCAGTTTCCCGCCCC G-3′	5′-GTGGGGGAGGGGCGT TCT-3′	5′-CTTTTCTGGTCCCGCG GGTAGG-3′
	5′-GGCCGGTAAAGGCCG AATAG-3′	5′-CTGCAACGCCTGTG T CTACG-3′	5′-ACATCCCTGCCCACAT ACGC-3′	5′-GGGGTTGGTGTAAAA TAGGGC-3′	5′-ATTTTACACCAACCCC CAGTT-3′	5′-CCACCTGGCCTATTTT ACACCA-3′
PCR conditions						
$MgCl_2$ concn (mM)	2.5	3	1.5	4	3	3
Amt of *Taq* polymerase (IU)	4	2.5	3	4	1.5	1.5
Annealing temp (°C)	54	64	63	62	58	59
Extension time (s)	45	30	45	90	30	30

[a] *L. infantum*, *L. chagasi*, and *L. donovani*.
[b] kDNA, kinetoplast DNA.

DNA hybridization step did not improve sensitivity compared with direct visualization of the gels under UV light. Finally, for all primer pairs, the intramethod reproducibility was high with both the *Leishmania* SDA and seeded dog blood samples, including with low parasite DNA concentrations, and even for artifactual bands.

On the whole, using these criteria, method Deb8-Ajs3 was thought not to be adequate for further evaluation. The five remaining primers were assessed for the diagnosis of CVL using dog peripheral blood.

Diagnosis of CVL by different PCR assays. Peripheral blood was drawn from 24 dogs living in an area of endemicity and was grouped according to clinical status and specific serology. Two groups were considered for the study: 17 dogs presenting typical CVL, i.e., with clinical signs and positive serology and a positive direct examination in the six cases where it was performed (group 1) and 7 asymptomatic dogs with positive serology (group 2). The results obtained with the different PCR methods for *Leishmania* detection are shown in Tables 3 and 4. The sensitivities of the different methods with dog clinical samples correlated well with the assessment previously made with artificial samples. Unexpectedly, however, primers Pia3-Pia4 yielded very poor results even in group 1, partly due to a high rate of PCR inhibitions which could not be reversed by any means (Table 3). For this reason, they were not tested further in group 2. Methods K13A-K13B and RV1-RV2 were the most sensitive in both groups of dogs. Again, whichever primer pair was used, systematic Southern blot analysis did not improve the sensitivities obtained after simple electrophoresis. The PCR results, as well as the specific serological tests, were all negative in the control group of 10 dogs living in a area where the disease was not endemic.

DISCUSSION

Several studies have shown the interest of PCR for the detection of CVL (2, 4, 18, 31, 35, 40, 43). Nevertheless, each study used a single PCR method and different types of samples; to our knowledge, only one comparative PCR study has been carried out for *L. infantum* (19), making the choice of an adequate assay difficult. Moreover, this PCR diagnosis remains awkward for a number of technical reasons. Here, our primary objective was to set up a reliable PCR assay using peripheral blood to detect *Leishmania* in dogs. Indeed, this type of sampling has the advantages of being straightforward, stress free, and easily repeatable compared with bone marrow, skin, or lymph node sampling. We also chose to work with the buffy coat portion, as *Leishmania* is an obligatory intracellular parasite and this approach has been validated in humans (11). Nevertheless, the utilization of dog blood proved difficult, and certainly more so than human blood; in particular, the lysis and extraction methods had to be specifically optimized in order to reduce the inhibition rate and improve the specificity of the reaction.

With the aim of finding the most efficient method for detecting the parasite in dogs, we compared six PCR methods and assessed their sensitivities, specificities, practicability, and overall reliability. All methods detected less than one parasite per reaction tube, but the sensitivity varied considerably according to the method used: by a factor of 500 with purified

TABLE 2. Results of comparative tests of the six PCR methods using artificial samples

Characteristic	PCR method					
	R221-R332	Pia1-Pia2	Pia3-Pia4	Deb8-Ajs3	K13A-K13B	RV1-RV2
Sensitivity of PCR[a]						
Pure culture (SDA)	0.05	0.01	0.01	0.01	0.0001	0.0001
Seeded blood samples	0.5 p/t (5 p/ml)	0.2 p/t (2 p/ml)	0.2 p/t (2 p/ml)	1 p/t (10 p/ml)	10^{-4} p/t (10^{-3} p/ml)	10^{-4} p/t (10^{-3} p/ml)
Artifactual PCR products[b]	±	+++	++[c]	++++	++	±
Ease of interpretation[d]	+++	+	+++	−	++	+++

[a] The parasite detection thresholds were determined as the highest parasite or parasite DNA dilution yielding ≥3 out of 4 positive reaction tubes: the sensitivity is expressed in parasites or parasite DNA equivalents (p) per tube (t) as well as per milliliter of blood with seeded blood samples.
[b] Presence of artifacts observed with seeded blood samples on the gels: ±, rare; ++, frequent (10 to 50% of reactions); +++, extremely frequent (50 to 80% of reactions); ++++, unavoidable.
[c] With these primers, a competitive amplification was constantly observed, yielding a fragment of ~70 bp and reducing the efficiency of the specific reaction.
[d] Interpretation on electrophoresis gels prior to hybridization tested with seeded blood samples (not with purified culture parasites): straightforward (+++) or hybridization necessary in ≤10% of cases (++), approximately half of cases (+), or almost all cases (−).

culture parasites and by a factor of 10,000 with seeded blood samples. Three methods targeting genomic DNA showed similar sensitivities, detecting two to five parasites per ml of blood. Two methods targeting the highly repetitive kinetoplast DNA, K13A-K13B and RV1-RV2, proved extremely sensitive, both detecting 10^{-3} parasite per ml of blood. Interestingly, one method targeting kinetoplast DNA was less sensitive than genomic-DNA-based methods, showing that PCR sensitivity is not necessarily correlated to the degree of reiteration of the DNA target. This has also been noted by Reithinger et al. (33) with different kinetoplastic primer sets. On the whole, our sensitivities were higher than (or equal to) those reported by other authors using the same primer pairs (19, 23, 29, 30, 31, 33, 34, 39, 41). It should be stressed that these theoretical sensitivity assessments are not always reported for diagnostic PCR assays. Nevertheless, we believe that they are excellent indicators of the performance of the assay. Their results are obviously tempered by the necessity that at least one parasite be present in the biological sample; still, the chances of detecting one parasite in 5 to 10 ml of blood are greater with an exquisitely sensitive method (like RV1-RV2) than with a moderately sensitive method. It is noteworthy that in our hands the DNA hybridization step did not improve the sensitivity of the PCR assay regardless of the method used. This had already been noted in our previous study with primers R221-R232 (11) but contrasts with other reports where hybridization improved the sensitivity by a factor of 100 up to 10,000 (33). One possible explanation for this discrepancy may be that our thorough optimization of the reaction conditions truly improved the sensitivity and ease of interpretation from electrophoresis gels, making hybridization unnecessary; indeed, our sensitivity prior to hybridization was ≈200-fold better than that obtained by others with primers R221-R332 (33), with both the SDA and seeded blood samples. As regards specificity and practicability, method Deb8-Ajs3 was considered inadequate for *Leishmania* detection in dogs in view of the extreme frequency of artifactual PCR products. Pia1-Pia2 and Pia3-Pia4 were found impractical but were kept for further evaluation.

When tested in the detection of CVL using peripheral blood, the efficiencies of the different methods correlated well with those assessed with artificial samples, except that method Pia3-Pia4 suffered from too-frequent PCR inhibitions. The remaining genomic-DNA-based methods (R221-R332 and Pia1-Pia2) did not prove sensitive enough even for diagnosis of clinically ill dogs (Table 3). This came as a surprise, since primers R221-R332 have given excellent results for the diagnosis of VL in humans (11, 18, 27), with an almost 100% sensitivity using peripheral blood. This might be explained by yet-unknown differences in the pathophysiology of the disease that may exist between humans and the canine reservoir, particularly regarding the degree of parasitemia. This hypothesis is supported by the fact that the most sensitive methods (K13A-K13B and RV1-RV2) could detect *Leishmania* in 100% of these dogs (Table 3). As this work was designed for the study of CVL prevalence and as previous studies underlined the fact that PCR can detect seronegative dogs that would otherwise remain undetected (29, 31, 40), we searched for a maximal sensitivity for detecting both symptomatic and asymptomatic parasite carriers, whatever the serological result. For this purpose, the methods using kinetoplastic primers (K13A-K13B and RV1-RV2) proved best adapted, as they could detect the parasite in 100% of symptomatic and asymptomatic seropositive dogs (Tables 3 and 4). However, serology has proved inefficient

TABLE 3. Comparison of five PCR methods for the detection of *Leishmania* in symptomatic dogs with positive serology

Dog code	Result[a]				
	R221-R332	Pia1-Pia2	RV1-RV2	KI3A-K13B	Pia3-Pia4
Rey5	+	+	+	+	+
Gor18	+	+	+	+	+
Rey9	+	+	+	+	+
Rey12	+	+	+	+	−
Rey18	+	+	+	+	+
Rey23	+	+	+	+	−
Sml19	+	+	+	+	Inh
Sml20	+	+	+	+	Inh
Tal 11	+	+	+	+	+
Mal 12	−	+	+	+	Inh
Mal18	−	+	+	+	Inh
Gor15	−	+	+	+	−
Pdh4	+	−	+	+	Inh
Sml21	+	−	+	+	+
Mal8	+	−	+	+	−
Rey13	−	−	+	+	Inh
Mal13	−	−	+	+	Inh
Total positive (n = 17)	12	12	17	17	6

[a] Inh, reaction inhibition; +, positive; −, negative.

TABLE 4. Comparison of four PCR methods for the detection of *Leishmania* in asymptomatic dogs with positive serology

Dog code	Result[a]			
	R221-R332	Pia1-Pia2	RV1-RV2	KI3A-K13B
Gor3	+	+	+	+
Sml 13	+	+	+	+
Mal6	−	+	+	+
Pdh18	+	−	+	+
Gor6	−	−	+	+
Sml12	−	−	+	+
Pdh8	−	−	+	+
Total positive (n = 7)	3	3	7	7

[a] +, positive; −, negative.

for detecting CVL in a rather high (5 to 40%) proportion of cases with clinical signs (15, 29, 31, 40, 42), whereas PCR has been shown to detect *Leishmania* in both asymptomatic and seronegative dogs (4, 31, 40). In this respect, it is noteworthy that we also tested four symptomatic dogs with negative serology and found them positive by PCR with the kinetoplastic primers but not with genomic primers (not shown). Furthermore, when we tested nine asymptomatic seronegative dogs, methods K13A-K13B and RV1-RV2 both detected the parasite in five dogs while methods R221-R332 and Pia1-Pia2 were negative for all of them (not shown). Several points argue for these being true positives and not contaminations. (i) The specificity of both methods was 100%. Indeed, all negative control tubes remained negative during the course of this comparative study. Moreover, DNA hybridization was used systematically to test for the presence of spurious amplification products which could be mistaken for positives. (ii) The group of 10 negative control dogs were all found PCR negative. (iii) Peripheral blood samples from these five seronegative dogs were DNA extracted twice at different time intervals, and all extractions were found to be positive. We therefore believe that these highly sensitive PCR methods can detect authentic healthy *Leishmania* infection carriers not detectable by other means. The duration, constancy, and intensity of parasitemia in canine hosts is still largely unknown. The question of a "gold standard" to define *Leishmania* remains open to debate, but PCR should help in this definition. Our results, together with previous reports (4, 29, 31, 40), indicate that the proportion of asymptomatic *Leishmania* carriers in the dog population might be much higher than previously thought. Still, the evolution of these asymptomatic carriers into "resistant" or clinically ill dogs (26), as well as their role in the maintenance of endemicity, remains to be better understood.

On the whole, although the PCR detection of CVL using peripheral blood is not without difficulties, it presents considerable advantages. In particular, its high sensitivity compared with conventional methods allows the detection of asymptomatic (and sometimes seronegative) dogs, a better negative predictive value, and the possibility of less invasive samples and of improving longitudinal follow-up and posttherapeutic monitoring. In our opinion, the utilization of an extremely sensitive method (based on kinetoplast DNA primers) is a necessity for optimal detection of all *Leishmania* carriers. Nevertheless, this extreme sensitivity, allowing the detection of asymptomatic carriers, implies a reduced positive predictive value for clinically patent disease. On the other hand, genomic-DNA-based methods showed a better positive predictive value for the disease, but the sensitivity was not 100% in clinically ill dogs. Therefore, both extremely and moderately sensitive PCR methods are needed for the time being to better define the infection status. In CVL, as in other infections, it might be interesting to play with the differences in sensitivity among PCR methods; once sensitivities have been clearly determined (prior to the study), they should be taken into account when defining the objectives (diagnosis or prevalence studies) and interpreting the data.

ACKNOWLEDGMENTS

We are grateful to Florence Michel, Bounleth Sanichanh, Claudine Rouquairol, and Catherine Martinez for technical help in the PCR study. We also thank Patrick Lami for his essential technical help with dog sampling.

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